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Expression of *fringe* is down regulated by Gurken/Epidermal Growth Factor Receptor signalling and is required for the morphogenesis of ovarian follicle cells

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SUMMARY

Signalling by the Gurken/Epidermal Growth Factor Receptor (Grk/EGFR) pathway is involved in epithelial cell fate decision, morphogenesis and axis establishment in *Drosophila* oogenesis. In the search for genes downstream of the Grk/EGFR signal transduction pathway (STP), we isolated a number of genes that are components of other STPs. One of them is a known gene, called *fringe* (*fng*). *Drosophila fng* encodes a putative secreted protein that is required at other development stages for mediating interactions between dorsal and ventral cells via Notch signalling. Here we show that *fng* has a dynamic expression pattern in oogenesis and that its expression in specific groups of follicle cells along the anterior-posterior and dorsal-ventral axes is defined by the repression of *fng* by Grk. Interfering with *fng* expression using antisense RNA experiments resulted in a typical *fng* mutant phenotype in the wing, and malformed egg chambers and abnormal organisation of the follicle cells in the ovaries, revealing that *fng* is essential in oogenesis for the proper formation of the egg chamber and for epithelial morphogenesis. This has been confirmed by re-examination of *fng* mutants and analysis of *fng* mutant clones in oogenesis.

Key words: Signalling pathways, Follicle cells, Cell-cell interactions, Oogenesis, *fringe*, *Drosophila melanogaster*

INTRODUCTION

Intercellular communication is essential for cell determination, differentiation, cell division, migration and adhesion which lead to the assembly of multicellular organisms. Morphogenesis of the follicular epithelium in egg chambers of *Drosophila* has been described by King (King, 1970) and depends upon the continuing interaction between groups of epithelial cells and between follicle cells and germline cells (Schüpbach, 1987; Ray and Schüpbach, 1996). Two of the signal transduction pathways (STPs) involved, the Gurken/Epidermal Growth Factor Receptor (Grk/EGFR) and Notch/Delta (N/Dl) STPs, are believed to be essential for determination of the axes of the ovarian follicle and the subsequent embryo (Gonzales-Reyes et al., 1995; Roth et al., 1995; Ruohola et al., 1991). However, the mechanisms underlying the interactions between different STPs, and the changes in gene expression and cell behaviour in response to them, remain largely elusive.

At the tip of each ovariole of the paired *Drosophila* ovaries is the germarium, which has been divided into subregions according to the stage of assembly of the egg chamber (King, 1970; Mahowald and Kambysellis, 1980). When the clusters of germline derived cells (cysts) reach region 2b, a thin layer of somatic cells, the pre-follicular cells, envelope each cyst to form a convex lens-shaped group of cells (Mahowald and Kambysellis, 1980). Pre-follicular cells are derived from somatic stem cells lying at the region 2a/2b border and migrate inwardly to surround each cyst and form the egg chamber (Margolis and Spradling, 1995). A small subset of follicle cells near both the anterior and posterior poles of the egg chamber are first determined and cease division as cysts move through region 2b (Margolis and Spradling, 1995). Egg chambers then leave the germarium and enter vitellogenesis, moving towards the posterior of the ovariole as they mature into eggs. The polar follicle cells help pattern the oocyte, egg and embryo (Ruohola et al., 1991; Gonzáles-Reyes et al., 1995; Roth et al., 1995). The remaining follicle cells divide an extra 4 times to increase to 800-1200 cells. In a series of following follicle cell movements most of the follicle cells migrate over the oocyte to become columnar, leaving stretched follicle cells over the nurse cells. Later, anterior-lateral groups of follicle cells migrate towards the anterior and secrete the chorionic appendages.

Grk/EGFR signalling is involved in the determination of

follicle cell fates along the anterior-posterior (AP) and dorsal-ventral (DV) axes in two steps, which helps to set the polarity of both the egg and the subsequent embryo (Gonzales-Reyes et al., 1995; Roth et al., 1995). Firstly, it is required for the determination of posterior polar follicle cells and secondly, for the determination of anterior-dorsal follicle cells. A modulation of Grk/EGFR signalling is essential for further subdivision of anterior-dorsal cells into dorsal midline and lateral follicle cell fates, and this affects the eggshell but does not affect the oocyte (Wasserman and Freeman, 1998; Zhao and Bownes, 1999).

The determination and patterning of terminal follicle cells, including polar follicle cells and stalk cells, on the other hand, also requires N/Dl signalling (Xu et al., 1992; Ruohola et al., 1991; Larkin et al., 1996; Gonzales-Reyes and Johnston, 1998). Both *N* and *Dl* mutants can disrupt the AP polarity of the oocyte, as seen in *grk* and *EGFR* mutant ovaries, suggesting that both STPs are involved in the same process to establish the AP axis of the oocyte (Ray and Schüpbach, 1996). Other neurogenic genes like *neuralised*, *mastermind*, *cut* and *daughterless*, when mutated, cause egg chamber fusions or supernumerary germ cells in the egg chambers, mimicking some of the *N* and *Dl* mutant phenotypes (Jackson and Blochlinger, 1997; Cummings and Cronmiller, 1994). These neurogenic genes have been postulated to be a conserved 'cassette', which specifies somatic follicle cell fates in a similar manner to their role in the peripheral nervous system (Ruohola et al., 1991).

Germline expressed *brainiac (brn)*, another neurogenic gene, co-operates with EGFR expressed in the follicular epithelium to regulate follicle cell morphogenesis (Goode et al., 1992). Mutants of *brn* and *egghead* (*egh*), as well as *N*, show a loss of apical-basal polarity and follicular epithelial cells accumulate in multiple layers around the oocyte, leading to the proposal that these genes mediate follicular morphogenesis by regulating germline-follicular cell adhesion (Goode et al., 1996a). Thus the *brn* pathway may help to contribute specificity to an EGFR function during oogenesis (Goode et al., 1996b).

The *fringe* (*fng*) gene has been found to be crucial for N/Dl signalling, though it was not originally identified as a neurogenic gene. The *fng* gene encodes a putative secreted protein, and its function is required for the formation of the wing margin (Irvine and Wieschaus, 1994) and the eye equator (Dominguez and de Celis, 1998). Fringe exerts its effect in wing patterning by modulating the ability of Serrate and Delta to activate Notch (Kim et al., 1995; de Celis et al., 1996; Diaz-Bejumea and Cohen, 1996; Doherty et al., 1996; Speicher et al., 1994). This activates genes involved in wing growth and patterning on either side of the wing margin.

In this paper, we demonstrate that the *fng* gene is also essential for the morphogenesis of ovarian follicle cells at different stages of *Drosophila* oogenesis. The expression pattern of *fng* in follicle cells is regulated by Grk/EGFR signalling at three different stages of oogenesis. Disruption of *fng* function by in vivo produced antisense RNA causes defects in the follicular epithelium at each of the stages when it is expressed. These phenotypes are confirmed by producing mitotic clones induced in oogenesis. We propose that Grk/EGFR signalling may modulate N function by controlling *fng* expression in oogenic development.

MATERIALS AND METHODS

Stocks

Embryo hosts for injection were w^{118}. Wild-type flies were *OrR*. grk^{HK} and grk^{WG} (Schüpbach, 1987), *fs(1)K10* (Wieschaus et al., 1978) and N^{ts1} (Cangan and Ready, 1989) were obtained from the Bloomington Stock Center. Other fly strains were fng^{D4} (Irvine and Wieschaus, 1994), fng^{13} *FRT80/GD* and *y w* P{hs-FLP}; *tub-lacZ* FRT80/TM3, *Sb* (Dominguez and de Celis, 1998), *UAS-lacZ* (Brand and Perrimon, 1993), GAL4-*C532*, GAL4-*C606* and GAL4-*C709* (Deng et al., 1997). All flies were raised on standard cornmeal-yeast-agar medium at 25°C.

Generation of transgenic flies

The GAL4-*C606* line was used to initiate the cloning of the gene near the P insertion site in the genome. The flanking genomic DNA we isolated was then used to screen an ovarian cDNA library (STRATAGENE, λzap, a gift from L. Jan and Y. Jan), and two cDNAs (*pBST-C7S2* and *pBST-C7S4*) were cloned, sequenced and found to be *fringe*.

To construct *fng*-sense and *fng*-antisense lines, *fng* cDNA (pBST-C7S2) was cut with *Xho*I and *Not*I or *Xho*I and *Xba*I and these two DNA fragments, (the *Xho-Not* and *Xho-Xba* fragments), were cloned into corresponding restriction enzyme sites of the *pUAST* vector (Brand and Perrimon, 1993). Subsequent *Sma*I-*Xba*I and *Eco*RI-*Not*I fragments were ligated into *pCaSpeR-hs* downstream of the heatshock promoter, forming *pHS-fng* and *pHS-as-fng* constructs. The constructs were then introduced into w^{118} flies by P element mediated germline transformation (Spradling, 1986), resulting in the transgenic flies: *hs-fng* and *hs-as-fng*.

Heatshock treatment of *hs-as-fng* fly lines

This was done as recommended by Tian Hsu (Hsu et al., 1996) with the following modifications. Transgenic flies were cultured in milk bottles at 28°C for 4 days. Flies were heatshocked for 50 minutes at 39°C once for the first and fourth day, and twice for the second and third day. Six hours after the last heatshock, ovaries were dissected. The larvae were cultured at 28°C for 3 days, and heatshocked at 39°C twice a day for 2 hours.

Whole-mount in situ hybridisation of ovaries

The procedure using DIG-labelled DNA as a probe was performed as described (Tautz and Pfeifle, 1989) with the following modifications. After dissection, ovarioles were separated from each other while in fixation solution (4% paraformaldehyde in PBS). The ovaries were washed with EGTA/methanol (0.5 M EGTA, pH 8 and methanol, 1:9) for 3× 5 minutes (ovaries can be stored at –20°C for up to 2 years at this stage and rinsed once with EGTA/methanol before the next step) and then with PBW (0.1% Tween 20 in PBS). Proteinase K treatment (0.1 mg/ml PBS) lasted 1 hour at room temperature (RT), followed by rinsing with glycine (2 mg/ml PBS) and three washes with PBW. Ovaries were postfixed for 20 minutes and washed with PBW for 6× 5 minutes. After equilibration in PBW/DNA hybridisation solution (1:1) for 10 minutes, they were used for hybridisation. After hybridisation, ovaries were successively washed with hybridisation solution at 45°C for 20 minutes, with hybridisation solution/PBW (1:1) at 45°C for 2× 20 minutes and with PBW at 45°C for 20 minutes, followed by 5 washes with PBW at RT. Ovaries were stained as described in the DIG detection kit from Boehringer Mannheim. The alkaline phosphatase-conjugated anti-digoxigenin antibodies were preabsorbed with ovaries that were treated as described above, before hybridisation.

The procedure using DIG-labelled RNA probes was performed as described by Wilkie et al. (1999).

DAPI and β-galactosidase staining

Ovaries were dissected in Ringer's solution and then fixed in 4%

paraformaldehyde in PBS for 20 minutes. After rinsing with PBS, the ovaries were stained with a nucleus-specific dye, DAPI (0.2 μg/ml in PBS), for 1 hour. For β-galactosidase staining, dissected ovaries were directly transferred into staining buffer (0.2% X-gal, 10 mM phosphate buffer, pH 7.0-7.6, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$ and 3.3 mM $K_4Fe(CN)_4$ and stained for between one hour and overnight. The ovaries were then washed with PBS for 3× 5 minutes, mounted in 80% glycerol in PBS and examined using Nomarski or epifluorescence optics.

Induction of mitotic recombination by FLP/FRT system

The fly lines *fng*^{13} FRT80/GD and *y w* P{hs-FLP}; *tub-lacZ* FRT80/TM3 *Sb* were crossed and their non-*Tubby*, non-*Stubble* progeny (*y w* P{hs-FLP}; *fng*^{13} FRT80/*tub-lacZ* FRT80) used for subsequent heatshock experiments (Dominguez and de Celis, 1998). Mitotic recombination in these flies results in homozygous cells carrying 2 copies of *fng*^{13} (white) or 2 copies of *tub-lacZ* (dark blue) in a heterozygous (blue) background. Two-day-old flies were yeasted overnight at 28°C, before being subjected to a 1 hour heatshock at 39°C. Flies were then left to age at 28°C.

Embryos laid by heatshocked flies were collected at 12-hour intervals on agar plates supplemented with apple juice (per 400 ml: 9 g agar, 10 g sucrose, 100 ml apple juice, in dH_2O) and aberrant phenotypes were scored. The embryos were left to age for a further 24 hours and the number of unhatched eggs counted before mounting in Hoyer's medium and viewing with dark-field microscopy.

Ovaries from heatshocked flies were dissected in *Drosophila* Ringer's solution at 10-hour intervals, fixed in 0.5% glutaraldehyde in PBS, washed in PBS to remove excess fixative, and stained in X-gal solution at 37°C until a blue colour developed. Ovaries were then mounted in 80% glycerol/PBS, and viewed with Normarski optics. White patches denoted clones of *fng*$^{-/-}$ cells.

RESULTS

Isolation of a *P[GAL4]* insertion next to the *fng* gene

During an analysis of a series of *P[GAL4]* insertion lines for expression in subsets of follicle cells (Deng et al., 1997) we selected a line, *C606*, which shows expression in anterior and posterior follicle cells from very early in oogenesis (stage 2 of King, 1970) then later in patches of follicle cells over the oocyte (Fig. 1A). We cloned the flanking genomic DNA 5′ and 3′ of the insertion and used it to isolate a cDNA with a related expression pattern. As with many lines the *P[GAL4]/UAS-lacZ* expression pattern represented part, but not all, of the expression pattern of the cDNA. This is presumably because it is influenced by some, but not all, of the regulatory elements. The *P[GAL4]* insert proved to be in the regulatory region, rather than in the coding sequence. Using the cDNA for in situ hybridisation to RNA we found no detectable expression in germ cells at any stage of oogenesis, however, expression in mesoderm-derived cells dramatically changes during oogenesis (Fig. 1B). The gene is first expressed in the follicle cells of region 2b of the germarium at about the time when follicle cells interleave cysts (Fig. 1C). The follicle cells are organised in a cup shape and surround the cluster of germ cells posteriorly. Its expression is then restricted to two groups of anterior and posterior polar follicle cells until stage 9 of oogenesis.

Between stage 8 and 9, there is some expression in the follicle cells surrounding the nurse cells as they migrate posteriorly towards the oocyte (Fig. 1D); expression is maintained in the anterior polar follicle cells as they migrate to become border cells and in the posterior polar follicle cells. Then at stage 9/10 expression occurs in all the follicle cells surrounding the oocyte except those in an anterior-dorsal location that receive the Grk signal from the oocyte, which determines the identity of the anterior-dorsal follicle cells. Thus it is expressed in a broad area of follicle cells ventrally and at the posterior of the oocyte (Fig. 1E). This is a potentially important gene as it is expressed in the cells that do not receive the Grk signal as well as having an earlier dynamic expression pattern in the follicle cells.

We therefore sequenced the cDNA and found that it was *fng* (Irvine and Wieschaus, 1994). This gene is required in imaginal discs of *Drosophila* to define boundaries, such as the wing margin (Irvine and Wieschaus, 1994) and the eye equator (Dominguez and de Celis, 1998). Its vertebrate homologues are used similarly to define limb outgrowth (Laufer et al., 1997; Rodriquez-Esteban et al., 1997; Johnson et al., 1995). Its role in oogenesis has not been investigated as there are no female sterile alleles described; the known mutants are either lethal earlier in development or, those of a few weak alleles, have wing defects.

grk regulates the expression pattern of *fng* in the follicle cells

In the wing disc, *fng* and *apterous* (*ap*) are expressed in the same cells and *fng* is presumed to be downstream of *ap* (Kim et al., 1995). We investigated, therefore, the expression pattern of *ap* in the ovary by in situ hybridisation to RNA. It is expressed in the germ cells but was not detected in the follicle cells that express *fng*, although very low levels of expression in these cells cannot be ruled out (data not shown). Thus *fng* is unlikely to be regulated by *ap* in oogenesis.

We have shown that *fng* expression in mid-stage egg chambers is in follicle cells that do not receive the *grk* signal (Fig. 1E). So, we investigated the expression of *fng* throughout oogenesis in *grk* mutants. We found that *fng* was expressed in more follicle cells in the early germarium (Fig. 2A), and its later expression patterns were altered in *grk* mutant ovaries (Fig. 2B,C). The tight localisation of *fng* to the posterior polar follicle cells at stage 9 was lost and a larger domain of posterior follicle cells expressed *fng* (Fig. 2B). So, lack of a Grk signal expanded the domain of *fng* expression. In *grk* mutants, the polar follicle cells located at the posterior of the oocyte do not become specified to take on a posterior identity and have the same identity as anterior polar follicle cells (Gonzales-Reyes et al., 1995). The anterior flanking follicle cells normally express *fng* and become nurse cell-associated follicle cells at stage 9 (Fig. 1D) but by stage 10 they no longer express *fng* (Fig. 1E). In the *grk* mutants it is possible that the expanded posterior expression at stage 9 could be because these flanking cells have also switched fate to become anterior cells and are equivalent to the nurse cell-associated follicle cells that express *fng* (Fig. 2B). Overall the results indicate that the Grk signal is required to repress the expression of *fng* in posterior flanking follicle cells in normal oogenesis. Somehow, it does not repress *fng* in the posterior polar follicle cells themselves and this suggests that the posterior polar cells and the flanking posterior cells respond differently to the *grk* signal at stage 9.

Later, expression of *fng* in stage 10 *grk* egg chambers is observed in all the anterior follicle cells around the oocyte (Fig.

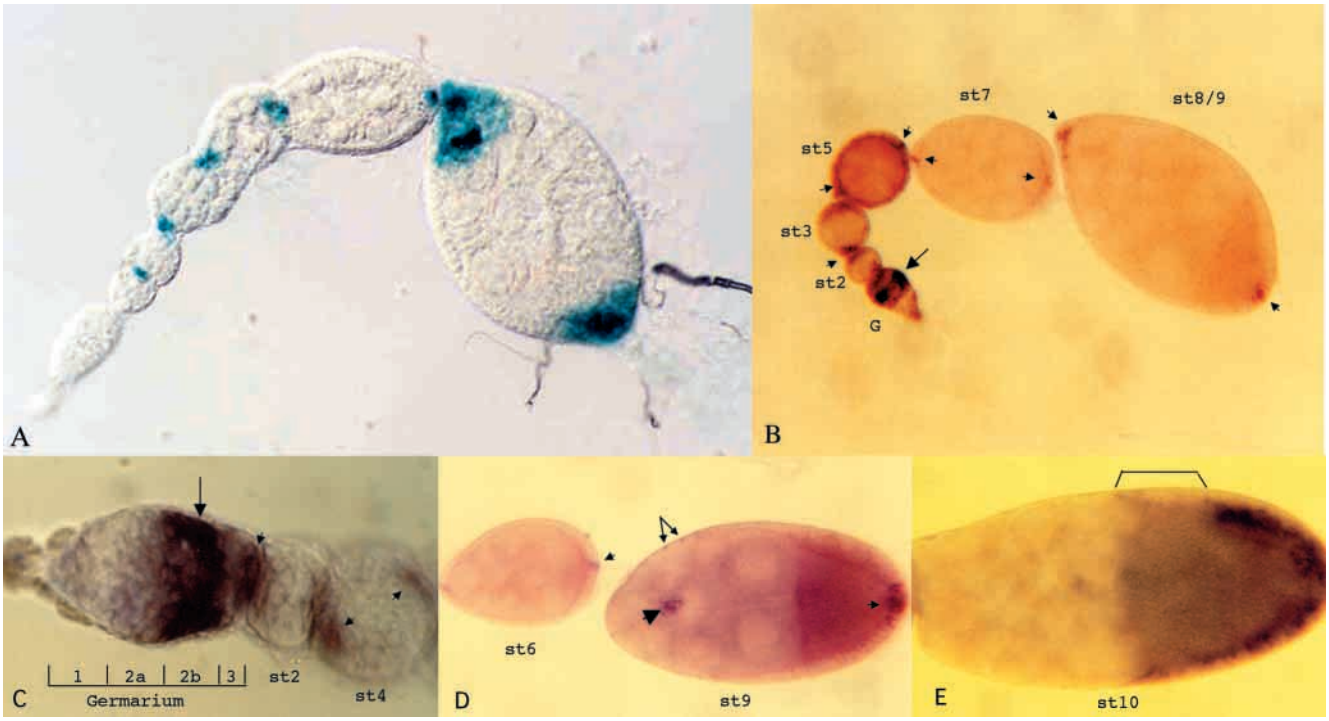

Fig. 1. Expression of *fng* and the *lacZ* reporter gene. (A) The β-galactosidase staining pattern of ovarioles from a *C606/UAS-lacZ* cross. C606 contains a P-element insertion close to the *fng* gene. (B-E) The distribution of *fng* transcripts in wild-type ovaries, as determined by in situ hybridisation to RNA in ovary whole mounts. (A) β-galactosidase staining in both anterior and posterior follicle cells. (B) *fng* expression in early oogenesis, from the germarium to stage 8/9. Note the strong expression of *fng* in the germarium (arrow) and the expression in both anterior and posterior follicle cells from stages 2-4, expression in all follicle cells at stage 5, and expression in polar follicle cells from stage 7-9 (arrowheads). St, stage; G, germarium. (C) There is strong expression of *fng* in the follicle cells in region 2b of the germarium (arrow), as well as in the follicle cells at both ends of the egg chamber at stages 1 and 4 (arrowheads). (D) *fng* expression in both anterior and posterior follicle cells (arrowheads) at stages 6 and 9 is shown. The large arrowhead indicates border cells and the small arrows indicate stretched follicle cells. (E) *fng* expression in all follicle cells surrounding the oocyte, except the anterior-dorsal follicle cells (bracket) in a stage 10 egg chamber.

2C), instead of having the anterior-dorsal gap observed in wild-type ovaries (Fig. 1E). Thus, at this stage *fng* is repressed, directly or indirectly, by *grk* in the anterior-dorsal follicle cells at stage 10 in the wild-type ovary (Figs 2C, 1E).

The regulation of *fng* via *grk* is not likely to be the only pathway leading to repression/activation of *fng* as the pattern of expression of *fng* is more dynamic than can be attributed simply to *grk* signalling. In summary, *grk* appears to repress *fng* expression in follicle cells that receive its signal, except in the posterior polar follicle cells. However, these cells have previously been determined to follow a specific developmental fate, namely to be polar follicle cells, and have thus already embarked on a different developmental programme to the cells flanking them.

The investigation of *fringe* function using a GAL4/*UAS-antisense-fringe* approach

There is no reported female sterile mutant of *fng* available and the viable alleles with abnormal wings are fertile. To investigate the function of *fng* in oogenesis we created a *UAS-antisense-fringe (UAS-as-fng)* construct and generated transgenic flies. Several independent lines were investigated. To test that the antisense RNA was produced when crossed to *P[GAL4]* lines, we used single stranded RNA DIG-labelled probes, which could detect separately the sense and antisense RNA. When *UAS-as-fng* flies were crossed to the original *P[GAL4]* line *fng* insert, *C606*, and the ovaries were probed to detect the antisense RNA, we observed the typical *fng* expression pattern (Fig. 3A,B) in those egg chambers that developed normally, with the difference being that the RNA had a *nuclear* location rather than being cytoplasmic (Fig. 3C). However, the experiment with the probe that detected the sense strand indicated that there was still *fng* RNA located in the cytoplasm (data not shown). Thus, it is likely that the levels of *fng* antisense expression were not sufficient to entirely block *fng* function.

Abnormal egg chambers were observed in the ovaries that expressed the antisense RNA. Many oocytes developed normally suggesting that low levels of *as-fng* is not sufficient to block *fng* function. However, in some egg chambers there was a failure, at stage 9, of the follicle cells surrounding the nurse cells to migrate normally to the posterior to produce the columnar follicle cells around the oocyte. This ultimately led to the degeneration of these egg chambers (Fig. 3D). Similar results were observed using another *P[GAL4]* line, *C709*, which is expressed in all the follicle cells over the oocyte (Deng et al., 1997). These observations imply that high levels of *as-fng* leads to the termination of oogenesis around stage 9. Thus, this approach does not allow us to investigate the later effects of *fng*. Specific GAL4 drivers for each of the *fng* patterns of expression at different stages of oogenesis with very high expression levels are not available. Therefore, to investigate the

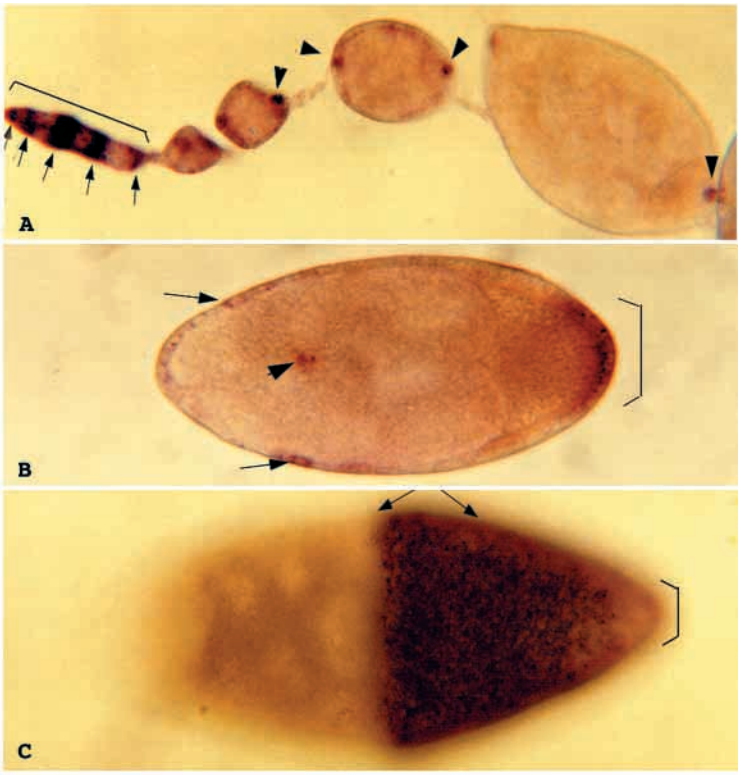

Fig. 2. Altered expression of *fng* in a *grk* mutant background. The expression pattern of *fng* RNA in *grk* mutant ovaries was dramatically changed. (A) In the elongated germarium (due to the failure of the egg chamber to pinch off) of *grk* mutant ovaries, the expression of *fng* was enhanced and/or maintained (arrows). However, *fng* expression in anterior and posterior polar cells in egg chambers from stages 2 to 8 was normal (arrowheads). (B) By stage 9, *fng* transcripts appeared in posterior flanking follicle cells in *grk* mutant egg chambers (bracket). Arrows and arrowhead indicate the normal expression of *fng* in stretched follicle cells and border cells, respectively. (C) In *grk* mutant egg chambers at stage 10, the expression domain of *fng* is expanded to the whole anterior-dorsal region indicated by a pair of arrows but is absent in the posterior region (bracket).

function of *fng* at different times in oogenesis we used a heatshock (hs) antisense approach.

The analysis of *fng* function using *hs-as-fng*

To investigate the function of *fng* at all stages of oogenesis we generated heatshock *antisense-fng* (*pHS-as-fng*) constructs and used them to produce several transformed fly lines. To test the function of the constructs, we heatshocked the *hs-as-fng* animals during larval development at the time *fng* is known to function in wing development. Approximately 10% of the flies that hatched had typical *fng* phenotypes with no wings or with abnormal wing outgrowths (data not shown). With a more severe heatshock regime this increases, but viability is reduced dramatically. Thus, the *hs-as-fng* is expressed at sufficient levels in imaginal discs to generate typical viable *fng* mutant phenotypes. The variability in wing phenotype is similar to that observed in viable *fng* mutants.

To follow the expression of the heatshock induced transcripts, RNA probes were prepared and hybridised to the RNA in ovary whole mounts at various times after heatshock. Antisense *fng* is not uniformly expressed in all transgenic flies after heatshock: it was induced in follicle cells from germarium region 2b to stage 10/11 and in posterior nurse cells from stage 9 to 10 in three *hs-as-fng* lines (*hs-7as2a*, *hs-7as2c*, and *hs-7as3*). But, in the *hs-7as4* line, *fng* antisense RNA was expressed in patches of follicle cells (data not shown). Fig. 4 shows an example of the expression of *fng* antisense RNA in transgenic flies after heatshock treatment. The results are repeatable and consistent within each transgenic line, suggesting the variability is related to the position of the insert in the genome. This means that heatshock experiments may not affect *fng* expression in all cells and the results must be interpreted with this in mind.

To investigate the function of *fng* in oogenesis we heatshocked females and observed the resulting ovaries at various times. This produced a number of abnormal phenotypes in the *hs-as-fng* ovary at different stages of oogenesis, including compound egg chambers, posterior disorganisation of follicle cells and disorganised anterior dorsal follicle cells, which are described in detail in the next section.

Early effects – *fng* is required for egg chamber formation

The earliest defect we observed in ovaries of *hs-as-fng* flies was a fusion of the egg chambers as they failed to pinch off normally from the germarium, generating large egg chambers with too many nurse cells (Fig. 5A). Between 2 and 5% of the egg chambers are fused. This is consistent with the early *fng* expression we observed in the follicle cells that surround the germ cells prior to producing the egg chamber. The large egg chambers derive from the fusion of two or more cysts. The nuclei in the germ cells at the anterior were often much smaller than those at the posterior region, presumably because they are from younger cysts. This phenotype was first described in *N* mutant egg chambers, and called compound egg chambers (Ruohola et al., 1991). We observed compound egg chambers with 2 oocytes, one at either end, with nurse cells between, or one at the posterior and one in the middle (data not shown). By staining for actin filaments and ring canals, we were able to count the number of cysts in the compound chambers. Mostly there were 2, but occasionally we observed 3 or 4 sets of ring canals, each representing a cyst, within a single compound egg chamber (Fig. 5A).

Expression of *fng* in a cap-like group of follicle cells that surround the cyst in region 2b of the germarium is consistent with the compound egg chambers that we observed in

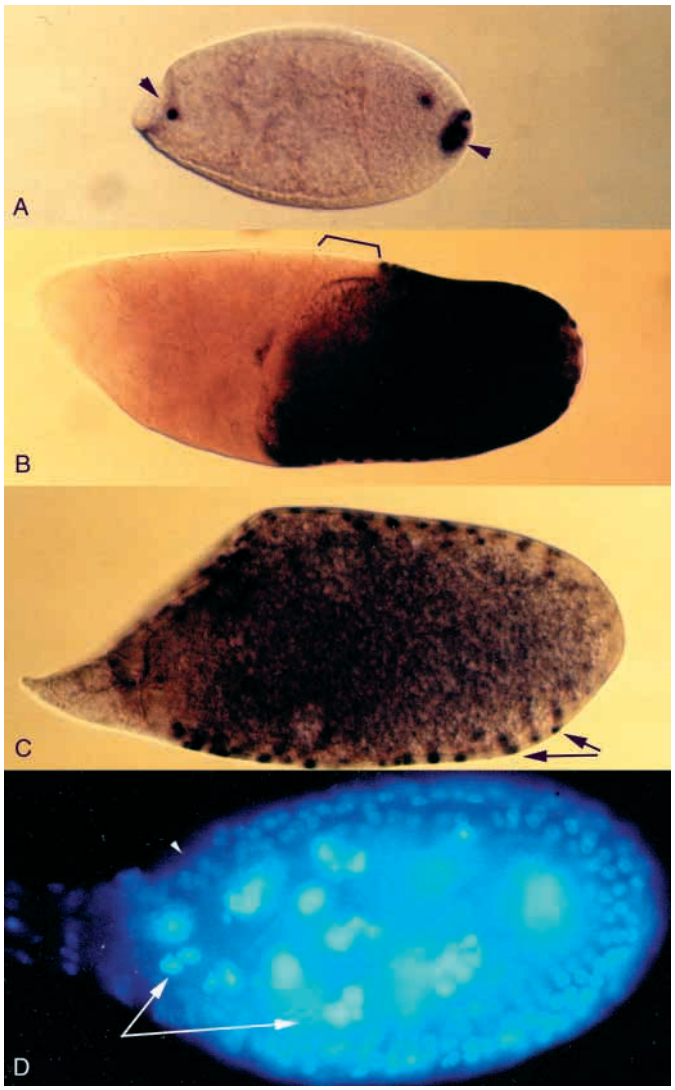

Fig. 3. Expression of *fng* antisense RNA and the phenotype of *UAS-as-fng/C606* offspring. All egg chambers are from ovaries of *UAS-as-fng/C606* heterozygous flies. The strong expression of *fng* antisense RNA is shown in A-C. The effect of *fng-as* experiment is shown in D. (A) The expression of *fng* antisense RNA in both anterior and posterior follicle cells at stage 8/9 (arrowheads). (B) The expression of *fng* antisense in follicle cells surrounding oocyte at stage 10, except the anterior-dorsal (bracket) and very posterior follicle cells. (C) The expression of *fng* antisense in all follicle cells at stage 12, except anterior and posterior follicle cells. Arrows indicate the nuclear localization of *fng* antisense RNA. (D) An egg chamber stained with DAPI. At the anterior, the dorsal appendage is visible. Inside, nurse cells start to degenerate (arrows). The surrounding follicle (arrowhead).

(antisense) fng mutant ovaries. This suggests that loss of *fng* products causes the failure of encapsulation and separation of individual cysts in the germarium. Mutations in the neurogenic genes, *N* and *Dl*, have similar defects. But the over-proliferation of polar follicle cells described for these mutations have not been observed in *fng* antisense ovaries. Using antibodies to fasciclin III, Notch and Cut, which mark the polar follicle cells, we saw no change in their staining pattern in *fng* mutant egg chambers, suggesting the fate of these cells is not altered by the loss of *fng* expression. This observation implies that *fng* and *Notch* are involved in distinct pathways that may regulate some overlapping aspects of oogenesis.

Thus, it seems that the main defect at this stage caused by reduced *fng* expression is an abnormal migration of the follicle cells resulting in incomplete assembly of each egg chamber unit.

Mid-stage oogenesis effects – *fng* function is required to control the behaviour of follicle cells

The *hs-as-fng* females produced oocytes with an abnormal multi-layered organisation of follicle cells. These cells were most often found in two locations. The first was at the posterior of the oocyte, where instead of the follicle cell layer being one cell deep it was often several cells deep at stage 8-9 (Fig. 5B). The second was the anterior-dorsal follicle cells, also displaying multilayered organisation during stage 10. However, multiple layers of follicle cells were seen at several positions in the egg chambers, and some egg chambers degenerate as is seen in GAL4/UAS-antisense experiments. This means that disruption of *fng* expression can lead to abnormal follicle cell behaviour and organisation during oogenesis.

The aberrant organisation of the posterior follicle cells occurs during stages 8 and 9 (Fig. 5B,D), covering the period when *fng* expression is observed in the anterior and posterior polar follicle cells. Sometimes there are multiple layers of follicle cells at both the anterior and posterior (Fig. 5D). To test if the abnormally arranged cells are posterior polar follicle cells or posterior flanking follicle cells we stained the *hs-as-fng* ovaries with anti-fasciclin-III antibody. This resulted in the staining of approximately the same number of anterior and

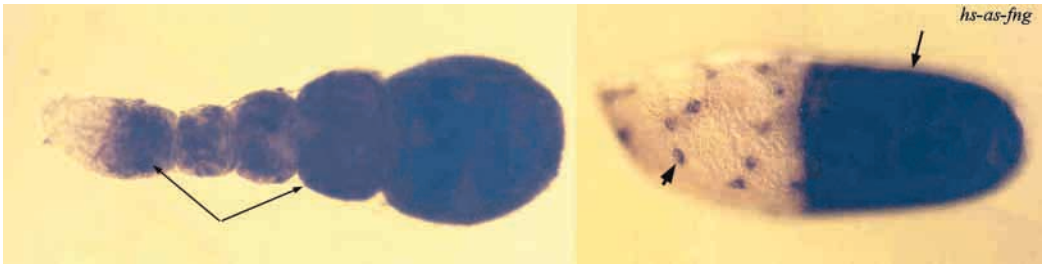

Fig. 4. Expression of *fng* antisense in *hs-as-fng* ovaries. After heatshock treatment, *fng antisense* RNA was expressed in both germline cells and follicle cells from region 2 of the germarium to stage 8 of oogenesis (small arrows) and then in stretched follicle cells (arrowhead), and all follicle cells surrounding the oocyte (large arrow) in ovaries from *hs-as-fng* females.

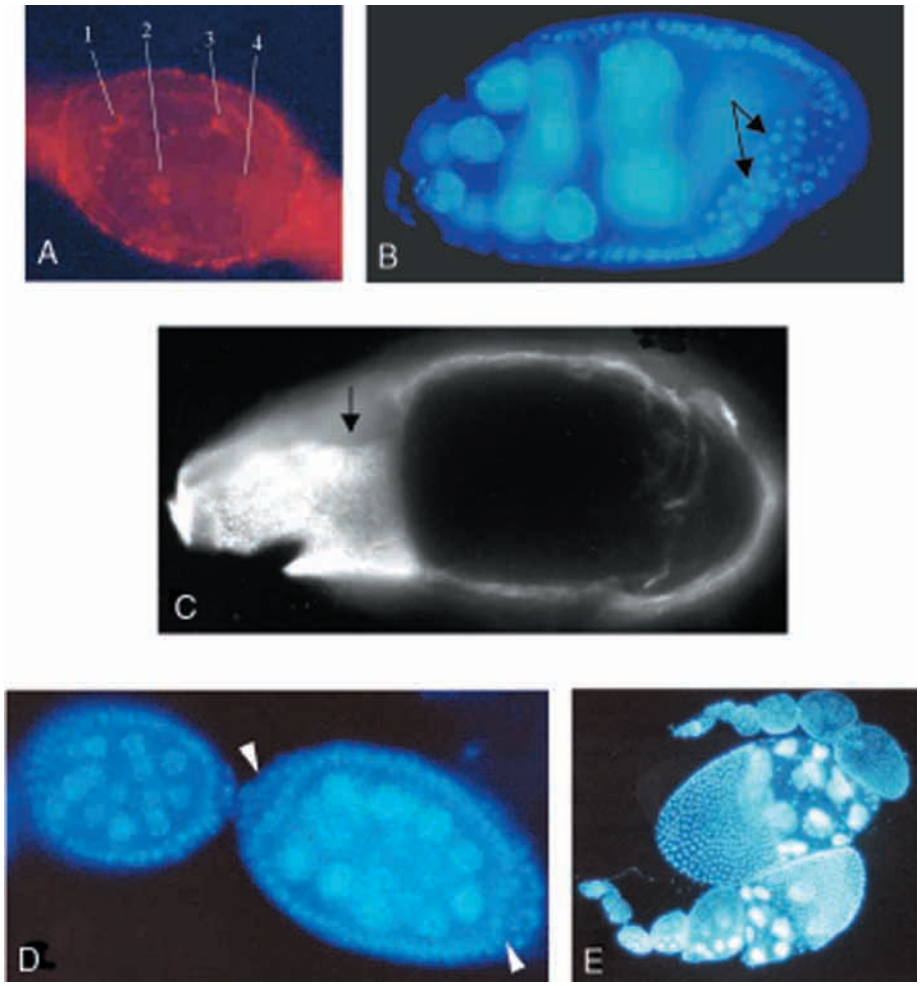

Fig. 5. Ectopic expression of *fng* antisense RNA causes abnormal oogenesis. Ovaries were stained with both DAPI (B,D,E) or Rhodamine-conjugated Phalloidin (A). Three different abnormal ovarian phenotypes have been found in *hs-as-fng* females. (A) Compound egg chamber composed of four cysts. Four sets of ring canals indicated by four arrows. (NB, the fourth set of ring canal is out of focus.) (B) Often there are disorganised multilayered follicle cells in the posterior region of stage 9 egg chambers (arrows). (C) By stages 10-11, the aberrant organisation of follicle cells in the anterior-dorsal region leads to the overproduction of dorsal appendage material (arrow) in abnormal locations. (D) Multilayered follicle cells (arrowheads) at the anterior and posterior of early (stage 8) egg chamber. (E) Wild-type egg chambers. A, B, D and E are epifluorescent micrographs. C is a dark-field micrograph.

posterior polar follicle cells as seen in wild-type, suggesting the additional cells do not originate from the posterior polar follicle cells that express *fng*, but those that flank them (data not shown).

We also saw disorganised follicle cell layers, which were no longer a unicellular layer with a tight bonding with the oocyte, and precise columnar cells in other locations. These included anterior-dorsal cells in some later oogenic stages. It is not clear if these arose because of a lack of *fng* in anterior-dorsal cells at stage 5 when *fng* is expressed in all follicle cells, or if a lack of *fng* in cells at the boundary between the *fng*-expressing and non-expressing cells at stage 10 is responsible. The latter case would demonstrate a non-autonomous *fng* function.

Late defects – *fng* is needed for eggshell differentiation

Abnormal migration of the anterior-dorsal follicle cells that secrete the chorionic appendages often occurs and shortened eggs with very abnormal chorionic appendages are produced (Fig. 5C). These presumably arise from egg chambers with weaker defects in the anterior-dorsal region, which are able to proceed with development rather than degenerating.

In summary, at several points in oogenesis disruption of *fng* leads to abnormalities in the follicle cell layer; cells lose their unicellular layered arrangement and become multilayered, the columnar cells often become more rounded and failures in cell migrations occur.

An analysis of *fng*$^{-}$ mitotic clones in oogenesis

Since antisense technology is still relatively novel, and we were only able to look at the effects of heatshock induced antisense RNA in detail, we generated clones of *fng*$^{-}$ follicle cells during oogenesis to investigate further the function of *fng* in those cells. Clones can only be induced if the cell subsequently divides, so, as cell division in the follicle cells ceases at stage 6, clones can only be induced prior to and including stage 5. This is a stage when all cells express *fng*. Thus, all clones are likely to have multiple effects on *fng* expression, and we cannot knockout expression such that it affects only the late borders between *fng* expressing and non-expressing cells by this method.

The phenotypes of egg chambers with *fng*$^{-}$ clones reflects the temporal variation in the *fng* expression pattern. Many egg chambers degenerate during stages 6-10, implying that the clones disrupt a function essential for the progression of oogenesis (Fig. 6B,D). Many compound egg chambers are formed, which then degenerate (Fig. 6C). These observations are consistent with the high frequency of degenerating oocytes at similar stages that were observed with the GAL4 directed UAS-*fng* antisense experiment, and fits well with the fact that many *fng*$^{-}$ clones will be induced early by this approach.

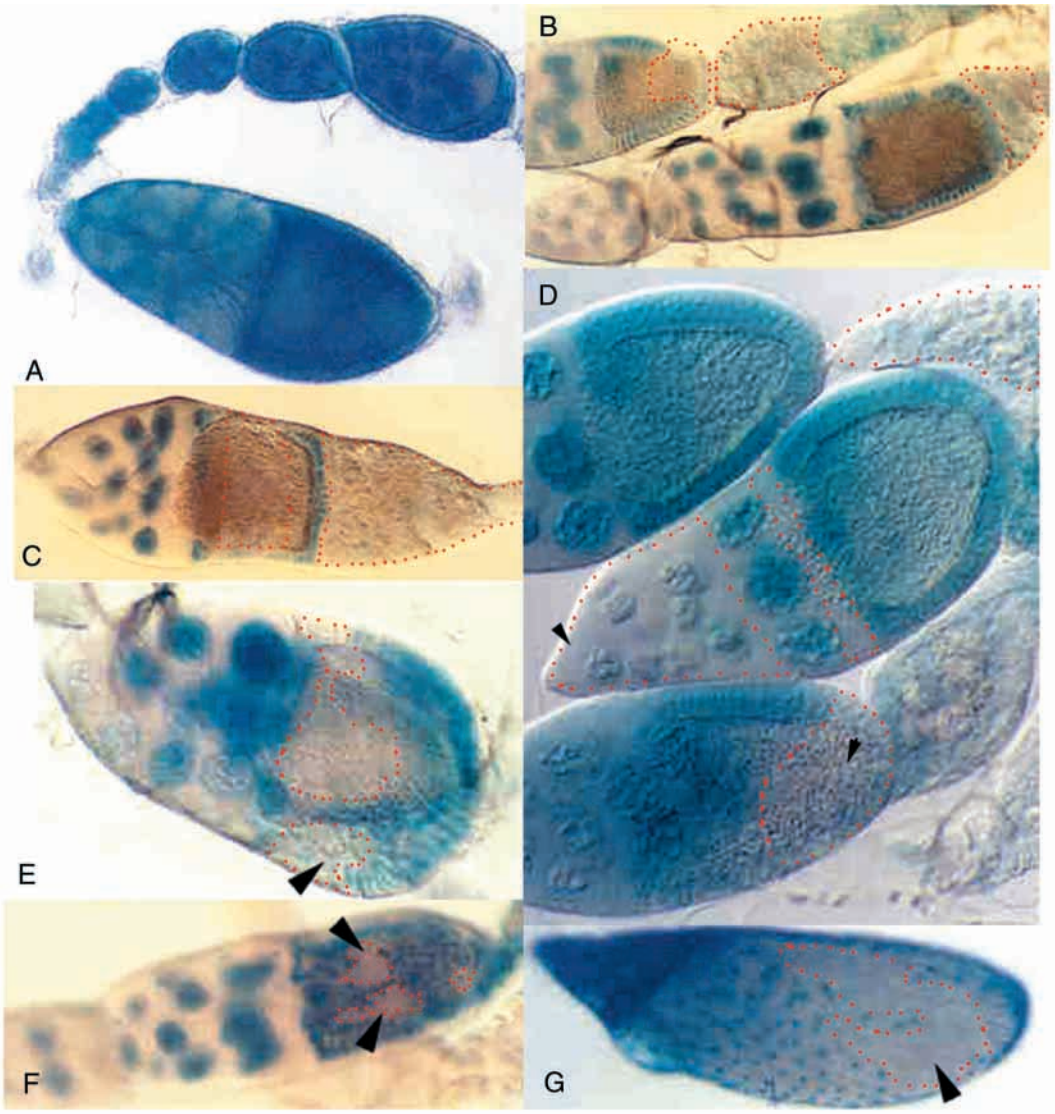

Fig. 6. Effects of fng⁻ clones in oogenesis. These photographs show the effect of inducing *fng*⁻ clones during *Drosophila* oogenesis. A shows that *lacZ* expression is driven by the tubulin promoter in all cells. This allows *fng*⁻ clones (demarcated by red dotted lines) produced by mitotic recombination to be distinguished as cells that have lost *lacZ* expression. (B) Degenerating egg chambers containing *fng*⁻ clones. (C) A compound egg chamber that contains a large *fng*⁻ clone, which will go on to degenerate. (D) 3 egg chambers at a similar stage of oogenic development. The top egg chamber has no *fng*⁻ clones and appears to be developing normally. The middle egg chamber appears to be unaffected by a germline *fng*⁻ clone (arrowhead). The bottom egg chamber appears to degenerate subsequent to this stage due to the presence of a posterior *fng*⁻ follicle cell clone (arrowhead). (E) An egg chamber with a disrupted follicular epithelium due to the presence of a large *fng*⁻ follicle cell clone (arrowhead). F and G show that some small *fng*⁻ clones (arrowheads) can be tolerated, and oogenesis can proceed to completion (G).

Clonal egg chambers that survive beyond stage 6 have a disrupted follicle cell epithelium; the follicle cells adopt a rounded morphology and delaminate from the oocyte beneath, often forming multilayers (Fig. 6E). Thus, the follicle cell layer becomes very fragile, and more prone to damage, than is observed in wild-type ovaries. Clones at more posterior locations of the egg chamber tend to be more deleterious. However, some larger clones can persist to later stages of oogenesis, suggesting that oogenesis can proceed even if a substantial number of follicle cells lack *fng* expression at stage 5. Additionally, some anterior/dorsal clones can be seen to have no effect on egg development, which is to be expected as *fng* is not normally expressed in these cells.

Some egg chambers that have only small clonal patches progress to the end of oogenesis (Fig. 6F,G). Presumably these egg chambers give rise to the egg phenotypes reported in the next section.

Eggs laid by mothers containing *fng*⁻ clones in the follicle cells

Once the egg has been laid, it is no longer possible to detect *fng*⁻ clones. This is a general problem in using mitotically induced clones to study egg development in *Drosophila* (Wasserman and Freeman, 1998). Three distinct dorsal appendage phenotypes are seen in the eggs laid by female flies with mitotically induced *fng*⁻ follicle cell clones (Fig. 7). In

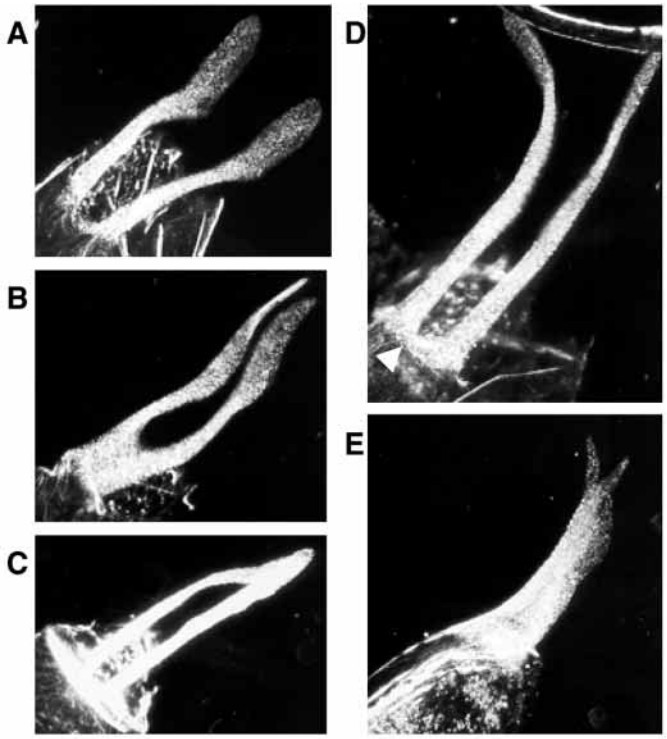

Fig. 7. Effects of *fng*$^{-}$ clones on dorsal appendage formation. The photographs show the dorsal appendage phenotypes of eggs laid by mothers containing *fng*$^{-}$ clones induced during oogenesis. (A) Wild-type (OrR) dorsal appendages. (B) Dorsal appendages that are fused at the base. (C) Dorsal appendages that are fused at the tips. (D) Dorsal appendages with a narrower dorsal gap (arrowhead). (E) Dorsal appendages that are fused along most of their length. These defects have no effect on the developing embryo, as the majority of the eggs hatch normally.

the time period 13-24 hours post-heatshock, approximately 8% of eggs have dorsal appendages that are fused along the base (Fig. 7B). Approximately 9% of the eggs laid between 13 and 36 hours have dorsal appendages that have a narrower dorsal gap between them (Fig. 7D). This phenotype is subtle and difficult to score, but is nonetheless real. A further 8-9% of the eggs laid between 25 and 36 hours have dorsal appendages that are fused at their tips (Fig. 7C). Subsequent to 36 hours post-heatshock, the percentage of mutant eggs drops. In all eggs laid by wild-type (OrR) females, only one displayed an abnormal phenotype. Thus, at any particular time interval, the frequency of aberrant dorsal appendages from OrR mothers was, at most, less than 3% of the total eggs laid. In all cases, these phenotypes do not appear to affect the embryos, as the majority of eggs (>87%) hatch normally. Thus, the late functions of *fng* seem to be related to the eggshell morphology but do not affect the embryonic axes. However, more analysis is required to exclude the possibility that the *fng*$^{-/-}$ clones are merely too small to affect embryonic development.

A re-examination of *fng* mutants

We decided to re-examine some of the *fng* alleles available. We were unable to produce homozygous *fng* females from *fng*^{E3} or *fng*^{1455}, but with *fng*^{D4} we obtained homozygous females. In *fng*^{D4} mutant ovaries, we observed both compound egg chambers and multilayered organisation of posterior follicle cells at stage 7-8, though the frequency is very low (approximately 2%).

Does *fng* affect the localisation of germline expressed transcripts?

Since *fng* is expressed in the posterior polar follicle cells, and these cells signal to the oocyte to re-orient the cytoskeleton and hence affect the anterior and posterior localisation of transcripts such as *bicoid* and *oskar*, we asked if the localisation of these transcripts was affected in *hs-as-fng* ovaries. We examined, therefore, the spatial and temporal expression pattern of *oskar* and *bicoid* RNA in the ovaries of heatshocked *hs-as-fng* flies. We saw no effect on *bicoid*, which localised normally at the anterior. We observed *oskar* at the posterior in both oocytes when egg chambers were produced with 2 oocytes in mirror image symmetry (Fig. 8B). The *oskar* RNA was sometimes more diffuse than in wild-type oocytes at later stages and did not localise as tightly at the posterior pole. We also observed *oskar* transcripts in the middle of the oocyte from stages 8-9 in some egg chambers. Therefore, reduced *fng* expression does affect the organisation of the oocyte and the posterior localisation machinery, but this is when egg chambers are likely to begin degeneration due to abnormalities in the epithelial layer. However, since *bicoid* transcripts localise normally and *oskar* RNA is normal at the early stages prior to degradation of follicles, it is unlikely that the abnormalities we see result from the failure of the cytoskeleton to repolarise. It therefore seems unlikely that *fng* is involved in the signal back to the oocyte from the posterior follicle cells that leads to repolarisation of the cytoskeleton and hence in the establishment of embryonic polarity.

Fig. 8. Distribution of *osk* transcripts in egg chambers from *hs-as-fng* females. (A) *osk* expression in early oogenesis (from germarium to stage 8/9) in a wild-type ovary. (B) The distribution of *osk* transcripts in a compound egg chamber at stage 8/9; arrows indicate the two oocytes at either end. This was from a *hs-as-fng* female.

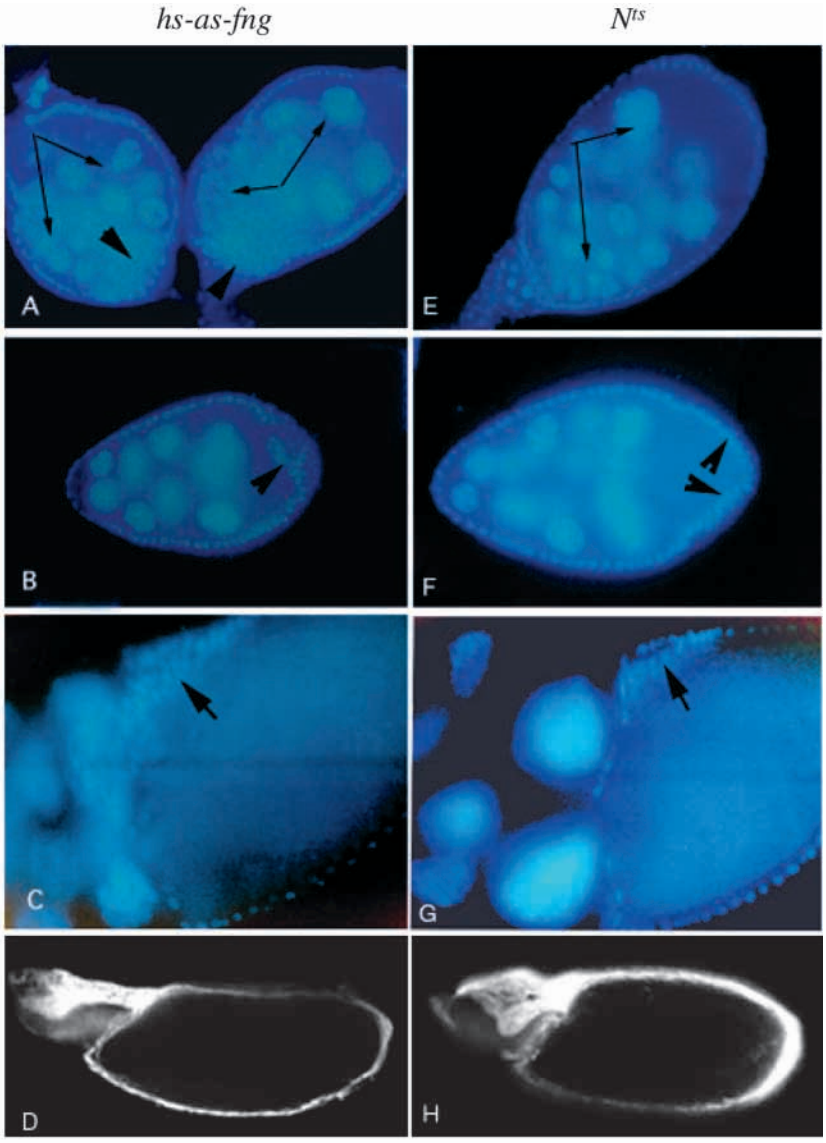

Fig. 9. N^{ts} and *hs-as-fng* have similar phenotypes. (A-D) *hs-as-fng* mutants. (E,F) N^{ts} mutants. (A,E) The compound egg chambers. Arrows indicate two different sizes of germline cells within 1 egg chamber. Arrowheads in A indicate the overproliferation of follicle cells. (B,F) The overproliferation of posterior follicle cells in both N^{ts} and *hs-as-fng* mutants (arrowheads). (C,G) The proliferation of anterior-dorsal follicle cells (large arrows). (D,H) The short, but enlarged dorsal appendage material compared with wild type (see Fig. 7A). (A-C,E-G) Epifluorescent micrographs; (D,H) dark-field micrographs.

Relationships between *fng* and the Notch/Delta signalling pathway

Some of the malformed egg chambers and abnormal dorsal appendages we observed in *hs-as-fng* ovaries were similar to those described in *N* mutant ovaries. In wing discs and in developing vertebrate limbs there is an interaction between Fringe and the Notch/Delta signalling pathway. Further, *N* has a role in cell proliferation and can cause overgrowth in the eye or thorax when an activated form of Notch is expressed. Thus the overproliferation effects may well be due to Notch interactions. However, one might expect that *fng* knockout would resemble gain-of-function *N* mutants rather than partial loss-of-function mutants. Further, there is no proliferation of follicle cells beyond stage 6, so the mid and late oogenesis expression of *fng* cannot be related to proliferation. Interestingly, in wild-type ovaries, *N* is strongly expressed in centripetal and anterior-dorsal follicle cells at stage 10B (Xu et al., 1992) whilst *fng* is expressed in the rest of the follicle cells surrounding oocyte. In other words, *N* and *fng* appear to have complementary expression patterns at some stages of oogenesis.

We re-investigated the phenotypes caused by a N^{ts} mutation during oogenesis to directly compare the phenotypes with those of *fng*. In N^{ts} females we observed all the ovarian phenotypes we had seen with the *hs-as-fng* flies. There were fused compound egg chambers in the early stages, abnormal organisation of posterior follicle cells and anterior-dorsal follicle cells, and abnormal chorionic appendages (Fig. 9). Most of these phenotypes matched those described by other authors (Ruohola et al., 1991), but the abnormal organisation of anterior/dorsal follicle cells we observed have not been described previously. Thus, *fng* mutants and N^{ts} mutants generate a similar set of phenotypic effects on oogenesis suggesting their related involvement in patterning the epithelial layer of follicle cells.

We investigated the expression of *fng* in N^{ts} mutant ovaries. Expression was essentially normal, including anterior dorsal gaps in *fng* expression in both oocytes when two were present in one egg chamber resulting from an egg chamber fusion (Fig. 10A-C). Thus *fng* and *N* may interact to co-ordinate different aspects of a shared event in oogenesis: *fng* is not downstream of *N*. This is true at other developmental stages.

In the wing disc, *fng* affects the expression of Serrate via its interactions with Notch. We used, therefore, a *P[GAL4]* insertion in the *Ser* gene and looked at the β-galactosidase staining pattern in ovaries when it was crossed to a *UAS-lacZ* line. It was not expressed in the follicle cells. Since it was possible that the β-galactosidase staining only represents a subset of the full *Ser* expression pattern we used in situ hybridisation and found no *Ser* RNA in the follicle cells. Thus, it is unlikely that *fng* regulates *Ser* in oogenesis, suggesting other molecules are needed for activating Notch in oogenesis.

These results suggest that, while *fng* interacts with the Notch signalling pathway as in the development of the *Drosophila* wing and the vertebrate limb, the details of this interaction seem to differ in several respects. Notch may be activated by different molecules and may play a different role in oogenesis to those studied in depth in neurogenesis.

DISCUSSION

The regulation of *fng* expression in oogenesis.

fng has a very dynamic expression pattern in oogenesis. It is

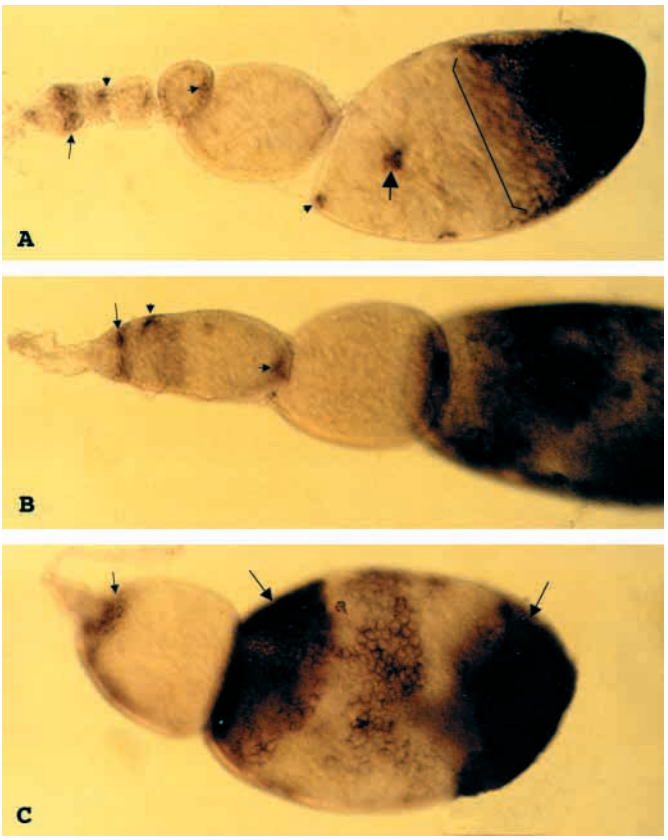

Fig. 10. Distribution of *fng* transcripts in N^{ts1} mutants. (A-C) Three typical classes of N^{ts1} ovarioles. (A) In morphologically normal ovaries, the expression of *fng* is normal. The small arrow indicates *fng* expression in the germarium. Arrowheads indicate its expression in anterior and posterior follicle cells. The bracket indicates the anterior-dorsal follicle cells without *fng* expression in the stage 10 egg chamber, where border cells (large arrowhead) migrate more slowly than posteriorly migrating follicle cells (large arrow). (B) In ovaries with a moderate phenotype, *fng* is still expressed in a small group of follicle cells, though mislocalised in the elongated germarium (arrowheads). Small arrow indicates *fng* expression in region 2b of the germarium. (C) In the ovaries with obvious phenotypes, there is a small group of follicle cells expressing *fng* at the junction between region 2b and the anterior of a compound egg chamber (small arrow). There is then another compound egg chamber with two oocytes at either end of the stage 10 egg chamber. Follicle cells surrounding the oocyte show strong *fng* expression (large arrows) except those presumably anterior-dorsal follicle cells.

first expressed in a cup-like group of follicle cells that will interleave the cysts to form the egg chambers and then in posterior follicle cells from stages 2 to 4. After a period of expression in all follicle cells, its expression is restricted to polar follicle cells, some of which become border cells. Finally, *fng* is expressed in all follicle cells surrounding the oocyte except in anterior-dorsal follicle cells.

Grk/EGFR signalling is used to define the expression pattern of *fng* at some stages of oogenesis. The posterior flanking follicle cells and the anterior-dorsal follicle cells, which do not express *fng*, define, in each case, the group of cells that receive the Grk signal from the oocyte. *grk* mutations lead to an expansion of the fng^+ expression domain, corresponding in time with the first signal to include the entire posterior follicle cell groups as well as the polar follicle cells. Later, coincident with the second Grk signal, expression expands to the anterior dorsal group of cells (Fig. 2B,C). We propose that there is a transcription factor that represses *fng* expression downstream of the Grk/EGFR signal pathway. The pre-determined polar follicle cells located at the posterior are somehow protected from this repression. We also noticed in *grk* mutants, that *fng* expression in the germarium was obviously expanded (Fig. 2A), suggesting that *fng* expression is also repressed by a Grk signal being received in some follicle cells in the germarium.

In the eye imaginal disc, *fng* is expressed in the ventral region and *mirror* (*mirr*), a gene encoding a homeodomain protein, represses *fng* expression in dorsal cells (McNeill et al., 1997). We have found that *mirr* functions downstream of the Grk/EGFR signalling pathway and negatively regulates *fng* expression in oogenesis (Zhao et al., 2000).

In the wing disc the expression of *fng* is regulated by *ap* (Kim et al., 1995). This is unlikely to be the case in oogenesis, as *ap* is expressed in the germline cells but not in the follicle cells at detectable levels.

Antisense RNA can disrupt normal gene function

Fng function is required in many developmental processes, so most *fng* alleles are lethal. However, we have shown that weak *fng* alleles disrupt oogenesis. *fng* is expressed in a dynamic pattern through oogenesis, and we have shown late expression (around stage 10) to be regulated by Grk/EGFR signalling. We have used overexpression of antisense *fng* RNA to examine the effects of downregulating *fng* expression in oogenesis.

We propose that antisense RNA binds to nascent transcripts in the nucleus, and prevents them from entering the cytoplasm. mRNA already present in the cytoplasm is not affected. Thus, this technique will only be effective for actively transcribed genes, which produce mRNAs and proteins with relatively short half-lives. High levels of antisense RNA appear to be required. Nevertheless, we have previously shown that targeted misexpression of antisense RNA downregulates specific protein expression in oogenesis (Deng et al., 1999). This technique allows more control over when target gene expression is affected compared to RNAi – driving expression by heatshock or by the GAL4/UAS system allows earlier lethal phases to be bypassed prior to inducing antisense RNA.

fng is required for the formation of egg chambers

fng is expressed in a cap of follicle cells at the time follicle cells migrate to surround the cysts. Disrupting this expression leads to follicle cells surrounding between 1 to 4 cysts of germ cells, which in turn leads to abnormal egg chambers. When a cyst of 16 germline cells moves to the 2B region of the germarium, epithelial cells derived from the epithelial precursor cells migrate posteriorly to interleave each cyst and form an egg chamber. In *hs-as-fng*, the follicle cells migrate, but fail to interleave each cyst (Fig. 5A). We found compound egg chambers when we induced clones of fng^- follicle cells early in oogenesis and we observed compound egg chambers in some *fng* viable mutants confirming the role of *fng* in this process.

It is not clear what role the germ cell cyst has in signalling its presence to the nearby follicle cells. It is possible that at this early stage, fng^- anterior follicle cells and fng^+ posterior cells

proliferate and migrate towards each other to surround the egg chamber. What initiates the transcription of *fng* in one set of follicle cells and not the other at this very early stage is unknown. The expanded expression of *fng* in the germarium of *grk* mutants suggests that Grk signalling may be used to repress the expression of *fng* in some follicle cells but not in others in the germarium.

fng in cell morphogenesis in the follicle cell epithelium

The relationship between oogenesis and the formation of wing outgrowths is not obvious, as the wing is in a flattened form and each egg chamber is spherical. The juxtaposition of fng^+ and fng^- follicle cells changes during oogenesis but, each time, they identify boundaries between cells known to take on different fates. In mid-oogenesis *fng* expression is observed in the posterior polar follicle cells but not the adjacent posterior follicle cells. This positioning is crucial for maintaining the integrity of the follicle cell layer. When the posterior polar follicle cells lack *fng*, the posterior follicle cells adjacent to the polar follicle cells are disorganised after forming multiple layers (Fig. 5B). Posteriorly positioned mitotic fng^- clones are particularly disruptive and lead to defects in follicle cell layers and degeneration of egg chambers. Thus, *fng* is important for the organisation of the follicle cell layer in that region and maintains it as a single cell epithelial layer around the oocyte. A little later, fng^+/fng^- defines the boundary between anterior-dorsal and posterior-ventral cells. Some egg chambers degenerate but some females lay eggs with abnormally shaped, enlarged dorsal appendages presumably due to the disorganisation of follicle cells in the anterior dorsal region (Fig. 5C). A few egg chambers of *fng* viable mutants show a similar abnormal organisation in the posterior follicle cell layer. Small clones of fng^- cells in the ventral region where *fng* is expressed late do not always disrupt oogenesis, so making a new boundary between fng^+/fng^- cells does not always cause defects. Although the larger earlier clones mostly lead to compound egg chambers and chambers with abnormal follicle epithelia which degenerate, the smaller clones presumably either do nothing or affect appendage development. We cannot relate clone positions to eggshell phenotypes as the follicle cells are degraded by the time eggs are laid.

Does *fng* function in the same way in oogenesis as at other developmental stages?

A family of *fng* genes has been found, initially in *Drosophila* and subsequently in the chick, mouse and humans, which are crucial in the formation of boundaries between adjacent groups of cells in many developmental processes. The original *Drosophila fng* gene was found to be crucial in margin determination and wing development, acting at the dorsal-ventral boundary in the wing imaginal disc (Irvine and Wieschaus, 1994). The chick gene, *Radical-fringe (R-fng)*, is needed for limb outgrowth, particularly at the dorsal-ventral boundary in the apical ectodermal ridge (AER) (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Three related *fringe* genes, *Manic*, *Radical* and *Lunatic Fringe*, have been cloned in mammals. These, too, are involved in boundary determination (Johnson et al., 1995). Their disruption normally leads to proliferation and outgrowth of layers of epithelial cells.

The expression of *fng* in specific subsets of follicle cells in oogenesis could indicate that *fng* boundaries are important in epithelial morphogenesis or, alternatively, *fringe* could have another function in oogenesis. The disruption of *fng* expression in either posterior or ventral follicle cells by expression of *fng* antisense RNA causes the aberrant organisation of follicle cells, as does inducing fng^- clones during oogenesis. A cup-like pattern of *fng* expression in the germarium, the failure of follicle cells to interleave each cyst, and the formation of a compound egg chamber in *hs-as-fng* ovaries, could indicate that there is a boundary required to separate the cysts to form the individual egg chambers and potentially could be stimulating proliferation of follicle cells at a fng^+/fng^- boundary at this stage. However, after stage 6 of oogenesis the mitosis of follicle cells ceases, so the fng^+/fng^- boundaries at this stage and beyond cannot be related to inducing outgrowths or proliferation. It seems that *fng* in the later stages is needed for the maintenance of an organised epithelial cell layer and may in some way play a key role in follicle cell migrations during morphogenesis possibly affecting cell adhesion. Its expression in posterior and ventral cells, or its lack of expression in anterior dorsal cells, is crucial for the correct morphogenesis of the chorionic appendages, which require accurate migration of two groups of follicle cells located within the non-*fng* expressing region. Exactly what role *fng* has at this stage, and which proteins it interacts with, remains to be investigated.

The relationship between *fng* and other genes in oogenesis

Grk/EGFR signalling has been found to be required for the establishment of a fng^+/fng^- boundary, which is essential for the morphogenesis of ovarian epithelial cells. In the *grk* mutant, this boundary is either shifted at stage 9 (Fig. 2B) or lost at stage 10 (Fig. 2C). It has been observed that *grk* homozygous ovaries have fused egg chambers (Goode et al., 1996b and our unpublished data), which is compatible with the overexpression of *fng* in the germarium of grk^- ovaries (Fig. 2A).

In wing development, the selective activation of Notch by Delta or Serrate along either side of the DV boundary, depends on the activity of Fng to set up the dorsal ventral boundary of the wing imaginal disc (Panin et al., 1997). In oogenesis, N/Dl signalling is necessary for the determination of polar follicle cells and the establishment of AP polarity of the egg (Xu et al., 1992; Ruohola et al., 1991; Bender et al., 1993; Larkin et al., 1996), indicating that this signalling is required at multiple stages in oogenesis. In N^{ts} mutant ovaries, there are compound egg chambers, mislocalization of posterior follicle cells and abnormal chorionic appendages (Xu et al., 1992; and this paper). All the ovarian phenotypes in N^{ts} mutants have been seen with the *hs-as-fng* flies (Fig. 5). The similar set of phenotypic defects in oogenesis generated by *hs-as-fng* and N^{ts} mutants suggests a relationship in patterning the epithelial layer of follicle cells in oogenesis. Nonetheless, Serrate has not been found to be expressed at a detectable level. If Fringe functions to modulate the activation of Notch by its ligands along either side of the boundary in epithelial morphogenesis (Panin et al., 1997), there should be another intermediate membrane bound protein, functionally equivalent to Serrate, present during oogenesis.

Although Fringe has been proposed to function as a

modulator of binding of the Notch receptor to its ligands, no receptor for it has been reported. Recent research has found significant similarities between Fringe-like proteins and *Drosophila* Brainiac and to the glycosyltransferases encoded by the *lex1* gene (Yuan et al., 1997). Unlike *fng*, *brn* is expressed in germline cells and is required for the morphogenesis of the follicular epithelium (Goode et al., 1996a). Surface glycosyltransferases can bind ligands and this requires association with the cytoskeleton for them to function as cell adhesion molecules (Shur, 1993). Mutants affecting the components of either the membrane or cytoplasmic skeleton cause compound egg chambers or alter follicle cell organisation, suggesting an important role for the cytoskeleton in epithelial morphogenesis (Lee et al., 1997, Dick et al., 1996 and Peifer et al., 1993). It is possible that Fringe also has a direct role in cell adhesion like Brainiac, and the disorganisation of the follicle cell layers we observe in *fng* mutant ovaries are consistent with this idea. The lack of *fng* expression in anterior-dorsal cells might enable those cells to migrate over the nurse cells to create the appendages.

Grk/EGFR signalling was originally found to be required for the establishment of both axes of egg and embryo (Schüpbach, 1987; Gonzales-Reyes et al., 1995; Roth et al., 1995). Recently, it was reported that N/Dl signalling, which is also involved in the development of the follicular epithelium, co-operated with *brn*, and may help to provide the specificity of Grk/EGFR function in oogenesis (Goode et al., 1996b). The observation that *brn* and *egh* are involved in both EGFR- and Notch-mediated epithelial morphogenesis has led to the proposal that the EGFR pathway is essential for establishing and maintaining posterior cell fates, while the neurogenic gene function acts subsequently to mediate adhesion of the follicular epithelium to the oocyte (Goode et al., 1996a). The relationship between EGFR signalling and the neurogenic genes is still not clear. The evidence for a direct link between them is that Grk/EGFR signalling is required to generate, via *mirr*, the expression pattern of *fng* (Zhao et al., 2000), which acts as a modulator of Notch signalling at other developmental stages. Since *fng*, *egh*, *brn* and *N* mutants all have similar mutant phenotypes, including compound egg chambers and multilayers of follicle cells (Xu et al., 1992; Rübsam et al., 1998; Goode et al., 1992; 1996b; Fig. 9 this paper), and both *brn* and *fng* encode proteins with a signal peptide and a central region similar to the glycosyltransferase (Yuan et al., 1997), it is possible that *brn* and *fng* have a similar function that provides the specificity for Notch signalling (Goode and Perrimon, 1997). The cytoskeleton has been recently found to be altered in *egh* mutant follicles (Rübsam et al., 1998). Further study on the interaction between glycosyltransferase-like proteins and the cytoskeleton in epithelial morphogenesis may help to explain how Fringe and Brainiac provide specificity to Notch signalling during oogenesis.

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Note added in proof

Fringe has now been confirmed as a glycosyltransferase (Maloney et al., 2000; Bruckner et al., 2000).

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